



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>A61K 39/395, C07K 16/46</b>		A1	(11) International Publication Number: <b>WO 97/33617</b> (43) International Publication Date: 18 September 1997 (18.09.97)
(21) International Application Number: <b>PCT/US97/03571</b> (22) International Filing Date: 11 March 1997 (11.03.97)		(74) Agents: APPLE, Randolph, T. et al.; Townsend and Townsend and Crew L.L.P., 8th floor, Two Embarcadero Center, San Francisco, CA 94111-3834 (US).	
(30) Priority Data: 08/614,584 13 March 1996 (13.03.96) US		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
(60) Parent Application or Grant (63) Related by Continuation US Filed on 08/614,584 (CIP) 13 March 1996 (13.03.96)		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(71) Applicant (for all designated States except US): PROTEIN DESIGN LABS, INC. [US/US]; 2375 Garcia Avenue, Mountain View, CA 94043 (US).			
(72) Inventors; and (75) Inventors/Applicants (for US only): QUEEN, Cary, L. [US/US]; 622 Benvenue Avenue, Los Altos, CA 94024 (US). SCHNEIDER, William, P. [US/US]; 200 Osage Avenue, Los Altos, CA 94022 (US). VASQUEZ, Maximiliano [CR/US]; 3813 Louis Road, Palo Alto, CA 94303 (US).			
(54) Title: FAS LIGAND FUSION PROTEINS AND THEIR USES			
(57) Abstract			
<p>Fas ligand fusion proteins comprising a polypeptide capable of specifically binding an antigen or a cell surface marker are prepared employing recombinant DNA technology for use in, e.g., treatment of autoimmune disorders.</p>			

***FOR THE PURPOSES OF INFORMATION ONLY***

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

5

## FAS LIGAND FUSION PROTEINS AND THEIR USES

### CROSS-REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of 08/614,584 filed March 13, 1996, the contents of which are incorporated by reference.

10

### FIELD OF THE INVENTION

The present invention relates generally to the combination of recombinant DNA and monoclonal antibody technologies to develop novel compounds for the suppression of T-cell mediated immune responses, including responses directed against the patient's own 15 tissues in autoimmune and inflammatory conditions or against transplanted tissues.

### BACKGROUND OF THE INVENTION

The Fas protein is a type I membrane protein that belongs to the tumor necrosis factor (TNF) receptor family (see S. Nagata et al., *Science*, 267:1449, 1995). Many 20 tissues and cell lines weakly express Fas, but abundant expression is found in the heart, lung, liver, ovary and thymus (R. Watanabe-Fukunaga et al., *J. Immunol.* 148:1274, 1992). In addition, Fas is highly expressed on activated lymphocytes including T cells (Nagata et al., *op. cit.*). Fas transmits a signal for apoptosis or programmed cell death (see C. Thompson, *Science* 267:1456, 1995) when it is triggered by binding of certain 25 antibodies such as APO-1 (B. Trauth et al., *Science* 245:301, 1989) and anti-Fas (S. Yonehara et al., *J. Exp. Med.* 169:1747, 1989). Apoptotic cell death is characterized by nuclear and cytoplasmic shrinkage, membrane blebbing, and degradation of chromosomal DNA in a characteristic pattern, and can be distinguished from necrotic cell death due to acute cellular injury (Thomson, *op. cit.*).

30

The natural ligand for Fas is known simply as the Fas ligand (FasL). Its rat (T. Suda et al., *Cell* 75:1169, 1993), mouse (D. Lynch et al., *Immunity* 1:131, 1994; T. Takahashi et al., *Cell* 76:969, 1994) and human (T. Takahashi et al., *Internat. Immunol.*

6:1567, 1994) forms have been cloned at the cDNA level. FasL is a type II membrane protein, i.e., having an extracellular carboxyl terminal domain and an intracellular amino terminal domain, and belongs to the TNF family of proteins (T. Suda et al., *op. cit.*).

The Fas ligand is strongly expressed on activated lymphocytes, in the testis (T. Suda et al., *op. cit.*) and the eye (T. Griffith et al., *Science* 270:1189, 1995), as well as on some cytotoxic T-lymphocyte (CTL) cell lines (Rouvier et al., *J. Exp. Med.* 177:195, 1993).

Transfected cells expressing FasL, as well as purified FasL protein (T. Suda and S. Nagata, *J. Exp. Med.* 179:873, 1994), are cytotoxic for cells expressing Fas. Thus, FasL transmits a signal for apoptosis by binding to Fas. More precisely, by analogy with the homologous TNF - TNF receptor system, whose molecular structure has been determined by X-ray crystallography (D. Banner et al., *Cell* 73:431, 1993), FasL is believed to function as a trimer. Also by analogy with TNF, the FasL trimer presumably binds one to three Fas molecules at the interface of respective FasL units (as schematically illustrated in Figure 1). Binding of two or more Fas molecules to a FasL trimer presumably causes dimerization of Fas, which transmits an apoptotic signal to the Fas-expressing cell.

Fas - FasL induced cytotoxicity is one of the two major mechanisms of CTL-mediated cytotoxicity (D. Kagi, *Science* 265:528, 1994). The Fas system is believed to play an important role in the clonal deletion of peripheral autoreactive T cells and in control of the immune response (S. Nagata and T. Suda, *Immunol. Today* 16:39, 1995; J. Dhein et al., *Nature* 373:438, 1995), as mice with inactivating mutations in Fas (*lpr* mice) or FasL (*gld* mice) develop generalized lymphoproliferation and autoimmunity.

In addition, it has recently been discovered that mouse testis tissue transplanted into allogeneic mice is not rejected, presumably because the FasL expressed on the Sertoli cells of the testis destroys activated Fas-expressing T cells that would otherwise attack the transplanted tissue (D. Bellgrau, *Nature* 377:630, 1995). Similarly, expression of FasL in the eye is sufficient to destroy infiltrating inflammatory cells and make the eye an "immune privileged" site with reduced susceptibility to immune response and inflammation (T. Griffith et al., *Science* 270:1189, 1995). Also, cotransplantation of allogeneic pancreatic islet cells with myoblasts expressing FasL in mice protected the islet cells from immune rejection (H. Lau et al., *Science* 273:109, 1996).

This ability of FasL to destroy activated T cells suggests that it has potential as an immunosuppressive drug. However, FasL is likely to be highly toxic when injected into animals and humans, because it will induce apoptosis of other cells expressing Fas in addition to T cells, for example liver cells. Indeed, an agonistic antibody to murine Fas 5 rapidly kills mice after intraperitoneal administration by causing massive necrosis of the liver, presumably mediated through apoptosis of hepatocytes via Fas (J. Ogasawara, *Nature* 364:806, 1993). Thus, compounds incorporating FasL that have specific cytotoxicity to autoimmune T cells and low non-specific toxicity are required for treatment of autoimmune disease and transplant rejection. The present invention fulfills 10 these and other needs.

#### SUMMARY OF THE INVENTION

The present invention provides novel fusion proteins that comprise a functional moiety of the extracellular domain of the FasL protein and a polypeptide capable of 15 specifically binding to a cell surface marker such as an antigen. The fusion protein may also comprise a linker, e.g., of from 8 to 40 amino acids in length, which may be from a human protein. The binding polypeptide can be an antibody, preferably a human or humanized antibody, and often of the IgG2 or IgG4 isotype. The fusion protein will preferably have reduced ability to cause the death of cells expressing the Fas protein, 20 relative to Fas ligand protein, but increased ability to cause the death of such cells when in the presence of the cells to which the binding polypeptide binds. In one embodiment, the FasL component will contain an amino acid substitution that reduces its tendency to form dimer, trimer, or other aggregates. DNA segments encoding the fusion proteins and cell lines producing them may be prepared by a variety of recombinant DNA techniques.

25 The fusion proteins may be utilized for the treatment of various autoimmune or other inflammatory conditions, including multiple sclerosis, rheumatoid arthritis, type I diabetes, inflammatory bowel disease, psoriasis, rejection of an organ transplant, or ischemia-reperfusion injury, as well as for treatment of cancer. For such use, the fusion proteins will be substantially pure and formulated in a pharmaceutically acceptable dosage 30 form. For treatment of a particular autoimmune disease, the binding polypeptide component of the fusion protein will bind preferentially to cells of the tissue under attack

in that disease, for example to neurons, pancreatic beta cells, synovial cells, or colonic epithelial cells.

#### BRIEF DESCRIPTION OF THE FIGURES

5       Figure 1. Schematic diagram of the presumed structure of the Fas - Fas ligand complex. Fas ligand (FasL) is presumed to form a trimer.

Figure 2. Schematic diagram of an Ig-FasL fusion protein, with the domains of the antibody labeled. S-S, disulfide bond; L, linker.

10      Figure 3. Schematic diagram of the mechanism by which an Ig-FasL or other FasL fusion protein apoptoses Fas-expressing cells in the presence of cells to which the protein binds ("binding cells").

15      Figure 4. Schematic diagram of the pVg2 expression vector, with key regulatory elements, coding regions, and restriction sites indicated.

Figure 5. Schematic diagram of the pVg2FasL expression vector used to express the heavy chain of the Ig-FasL fusion protein, with an antibody heavy chain variable 20 region (V<sub>H</sub>) inserted at the XbaI site. Key regulatory elements, coding regions, and restriction sites are indicated.

Figure 6. Nucleotide and amino acid sequence of the CH3/FasL domain of an Ig-FasL fusion protein. Amino acid positions 1 to 106 comprise C<sub>H</sub>3. Residues 107 and 25 108 result from the addition of a KpnI site. Residues 109 to 287 constitute the extracellular domain of FasL with a His to Ser substitution at position 139.

#### DETAILED DESCRIPTION OF THE INVENTION

In one aspect, the present invention is directed to a fusion protein comprising a 30 functional moiety of the extracellular domain of the FasL protein and a polypeptide capable of specifically binding to a cell surface marker, such as an antigen, expressed on

particular cell-type(s) or tissue(s). In a preferred embodiment, the binding polypeptide comprises the variable domain of an antibody. In a particularly preferred embodiment, the antibody is humanized, human or from another primate species. However, the binding polypeptide may also comprise, for example, the binding site of a cellular receptor; a receptor ligand such as a cytokine, lymphokine, interleukin, growth factor, hormone or the like; or the binding site of an adhesion molecule, such as a selectin or integrin. A large number of such binding proteins are known to those skilled in the art (see, e.g., Fundamental Immunology, 3rd ed., W. E. Paul, ed., Raven Press, 1993, which is hereby incorporated by reference). As used herein, the term "cell surface marker" refers to a carbohydrate, glycolipid, etc. but most often a protein which localizes to the plasma membrane of a cell and a portion of which is exposed to the extracellular region (e.g., an integral membrane protein or a transmembrane glycoprotein), wherein said extracellular portion can be specifically bound by an antibody or other ligand, i.e., with an affinity of stronger than about  $1 \times 10^6 \text{ M}^{-1}$ . The term cell surface marker also refers to a polynucleotide sequence encoding such a cell surface protein. Various cell surface proteins can be used as cell surface markers, including, for example, a CD (cluster of differentiation) antigen present on cells of a hematopoietic lineage (e.g., CD2, CD4, CD8, CD21, etc.),  $\gamma$ -glutamyltranspeptidase, an adhesion protein (e.g., ICAM-1, ICAM-2, ELAM-1, VCAM-1), hormone, growth factor and cytokine receptors, ion channels, and the membrane-bound form of an immunoglobulin  $\mu$  chain. Preferably, a cell surface marker protein is a protein which is normally expressed at significant levels on the cells to be treated, and may be selected for use in the methods and constructs of the invention on the basis of the practitioner's desired application. By definition, the recognition or functional moiety of the extracellular domain of the FasL protein preferably contains at least the determinants required to bind to the Fas protein and transmit an apoptotic signal under treatment conditions. Typically, FasL fragments comprised in the recognition domain will contain only a portion of the extracellular domain. Such fragments will preferably retain the binding specificity of an intact FasL polypeptide, but will be soluble rather than membrane bound. Preferably, the FasL component of the fusion protein is found within a segment of up to about 10, 25 or 50 amino acids within the FasL extracellular region.

An example of a preferred embodiment of the invention is diagrammed in Figure 2. Such an embodiment, in which the FasL domain is linked to an immunoglobulin (Ig), is denoted Ig-FasL. The illustrated immunoglobulin (antibody) may bind to any epitope on the surface of particular cells or tissues. The optional polypeptide linker (L) between 5 the C-terminal domain of the antibody and the FasL moiety is preferably made so as to allow the two FasL moieties in the dimeric molecule to themselves dimerize, and may contain from 1 to about 100 amino acids, preferably 8 to 50 amino acids and most preferably 12 - 35 amino acids. Examples of preferred linkers are the 34 extracellular 10 amino acids of FasL that are proximal to the membrane, possibly with one or more amino acid substitutions, or a part of this sequence, or the sequence (Gly<sub>4</sub>Ser)<sub>N</sub> where N is from 1 to 8, preferably 3 to 6. In preferred embodiments, the sequence of the linker will be essentially (i.e., at least about 75% but preferably 80%, 90%, 95% or more) identical to a sequence occurring in a natural human protein in order to reduce immunogenicity of the 15 fusion protein. The linker will generally have a sufficient number of hydrophilic residues to be adequately soluble in water, and may contain a significant number of glycines and/or prolines in order to give it the degree of flexibility or rigidity desired.

Such linkers may also be used to connect other types of binding polypeptides and the FasL moiety. The linker may be attached to the binding polypeptide or FasL moiety, or those domains attached directly, via non-peptide or non-covalent bonds, for example 20 via a disulfide bond, a chemical cross-linker, or leucine zipper peptides such as jun/fos (see, e.g., PCT/US92/10140 [WO 93/11162], which is incorporated herein by reference). Art-known chemical cross-linkers that can be suitable for this purpose include 25 homobifunctional linkers such as N-hydroxysuccinimide esters, e.g., dithiobis(succinimidyl propionate) (DSP), and heterobifunctional linkers such as N-succinimidyl-3-(2-pridyldithio)-propionate (SPDP) and other cross-linkers listed in the Pierce Chemical Company catalog or well-known in the art, which may be used according to the manufacturer's suggestions and recommendations or the art. The FasL domain will preferably be attached to the carboxy terminus of the binding polypeptide, but may also be attached to the amino terminus or elsewhere. Each monomer of Ig-FasL has two 30 polypeptide chains - an antibody light chain and a heavy chain/FasL fusion chain - but other FasL fusion proteins may have fewer or more chains.

In other preferred embodiments, one or more domains of the antibody molecule are deleted, for example the C<sub>H</sub>3 and/or C<sub>H</sub>2 domains, or these domain(s) and the hinge and/or C<sub>H</sub>1 domains. Alternatively, the antibody may be a single-chain antibody (see, e.g., Bird et al., *Science* 242:423, 1988, which is incorporated herein by reference) or 5 have only one domain or be bispecific (e.g., PCT/US92/10140, which is incorporated herein by reference). The resulting fusion protein may be dimeric or monomeric. In preferred embodiments, the antibody constant region is human, and the antibody is of the IgG class, especially IgG2 or IgG4 to reduce effector function, but possibly IgG1 or IgG3. However, constant regions from other mammalian, especially rodent or primate, 10 species may be used, as well as the IgD, IgM, IgA or IgE isotypes. Various amino acid substitutions, deletions and/or insertions may also be made in the antibody component. For example, one or more amino acids in positions 234 to 237 of C<sub>H</sub>2 may be substituted (using the numbering scheme of the human Eu antibody and counting from the amino terminus of the heavy chain), e.g., with alanine, to reduce or eliminate binding to the Fc 15 receptors (see commonly assigned US 08/656,586, wherein amino acids 234 and 237 of IgG2 are substituted with alanine). Substitutions in the antibody or FasL moiety may also be made to eliminate glycosylation sites, introduce or eliminate disulfide bonds, improve solubility or stability, or provide other desirable properties. The positions of some 20 glycosylation sites in FasL are given in Suda et al., op. cit. or may be determined from the sequence. Instead of using the natural Fas ligand protein in FasL fusion proteins, other "Fas ligand" proteins (polypeptides) that bind to Fas and transmit an apoptotic signal may be used, for example polypeptides comprising the variable domains of anti-Fas 25 antibodies such as APO-1, or novel polypeptides that bind to Fas found using phage display methods (see U.S. Patent 5,223,409, which is incorporated by reference).

In a preferred embodiment, the fusion protein will have reduced ability to cause the apoptosis of cells expressing the Fas protein (target cells), e.g., activated T cells, transfectant cells expressing Fas, or hepatocytes. That is, at least 2-3 times as much, preferably at least 10 or 100 times as much, and most preferably 1000 or more times as much fusion protein as soluble FasL protein or extracellular domain alone will be 30 required to induce a given amount of apoptosis or cell death (e.g., 50%, 75%, 90% or essentially 100%) in the target cells. On the other hand, in preferred embodiments, the

fusion protein will have greater effectiveness in causing the apoptosis or death of target cells, especially activated T cells or cytotoxic T lymphocytes (CTLs) or Fas-expressing transfectants or cancer cells, in the presence of other cells to which the binding polypeptide of the fusion protein specifically binds (the binding cells). That is, in the 5 presence of a sufficient number of binding cells (typically from about 0.1 to 1 or 10 times as many as target cells), at least 2-3 times less, preferably at least 10 or 100 times less, and most preferably 1000 or more times less, fusion protein will be required to induce a given amount of cell death in the target cells, relative to FasL protein, or relative to 10 fusion protein in the absence of binding cells. A fusion protein that has reduced ability to apoptose target cells relative to FasL, and increased effectiveness in the presence of binding cells, is said to be "specifically cytotoxic" for the target cells.

Without being bound by theory, the inventors believe that the fusion protein has reduced ability to apoptose target cells alone because within the fusion protein, the FasL moiety forms monomers or dimers and not trimers. This property can be further 15 enhanced by mutation of critical amino acids involved in the trimerization of FasL, determined by in vitro mutagenesis experiments or by analogy to the known structure of the TNF trimer. Such a monomer or dimer is expected to bind only a single Fas molecule, which is not sufficient to cause the dimerization or aggregation of Fas needed to transmit an apoptotic signal to the target cell. However, once the fusion protein has 20 bound to the surface of the binding cell, the simultaneous binding of multiple fusion protein molecules on the surface of that cell to Fas molecules on the target cell can aggregate those Fas molecules and induce an apoptotic signal with increased effectiveness, as illustrated in Figure 3. Similarly, the fusion protein has the ability to kill cells that express both the target of the binding polypeptide and Fas.

25 Thus, in one aspect the fusion protein of the present invention has a reduced ability, in vivo or in vitro, relative to soluble FasL protein or its extracellular domain, to cause death of cells expressing Fas protein. Moreover, the novel fusion proteins will also have an increased ability, in vivo or in vitro, to cause death of a first population of cells expressing Fas protein, when such first population of cells are in the presence of a second 30 population of cells to which the polypeptide binds, relative to the absence of such second population of cells.

As alluded to above, in a preferred embodiment of the invention, the FasL moiety of the FasL fusion protein comprises amino acid substitutions or mutations that further reduce its ability to form dimers, trimers or higher oligomers or aggregates, especially when in solution. This further enhances the desirable properties described above, e.g.,

5 decreases the ability of the fusion protein to kill cells expressing Fas, especially relative to soluble FasL protein or extracellular domain. However, the fusion protein with amino acid substitutions still has the ability to cause death of a first population of cells (e.g., cells within the first population) expressing Fas protein when such first population of cells are in the presence of a second population of cells to which the polypeptide binds, further

10 increased relative to the absence of such second population of cells.

Preferred amino acid substitutions for this purpose disrupt the subunit-subunit interface of FasL so as to disfavor formation of the trimer in solution, but without disabling the potential reconstitution of an aggregate state once the fusion protein has bound to a cell surface via its binding polypeptide component. Such substitutions should

15 preferably not affect the amino acids involved in binding of FasL to Fas. One approach to determining such substitutions is to align FasL with the homologous protein TNF- $\alpha$ . The alignment is, in turn, used to map structure-activity data collected in the TNF system to the FasL system, and also forms the basis for the construction of a 3-dimensional structure model of FasL and of the FasL-Fas complex based on the known crystal

20 structure of the TNF-TNF receptor complex (D. Banner et al., *Cell* 73:431, 1993, which is incorporated herein by reference), using homology methods well-known to those skilled in the art of molecular model building.

Based on this analysis, substitutions were made in the FasL polypeptide extracellular domain. Substitutions are described using the 1-letter amino acid code and

25 with the numbering referring to the position of the residue in the FasL sequence of Takahashi et al., *Internat. Immunol.* 6:1567, 1994 (which is incorporated herein by reference, and which is provided in Table 4). In the FasL sequence provided in Table 4, the extracellular domain extends from about residue 103 to the carboxy terminus. It will be appreciated that the FasL sequence of Table 4 is provided by way of illustration (e.g.,

30 to identify specific amino acid residues that may be advantageously mutated) and not by way of limitation. As used herein, when the fusion protein of the invention is derived

from naturally occurring FasL protein, the FasL protein may be a human FasL protein (including variants, e.g., allelic variants), a non-human FasL protein, or a FasL polypeptide containing deletions or insertions (e.g., from about 5 to about 20 amino acids or more), or substitutions (e.g., conservative substitutions) compared to a naturally occurring FasL sequence.

The substitutions H148S (i.e., substitution of histidine by serine at position 148), Y189A, Y192A, and Y244A, were derived from the analogous mutations shown to affect TNF- $\alpha$  structure (X.-M. Zhang et al., *J. Biol. Chem.*, 267:24069, 1992, which is incorporated herein by reference; these four substitutions also satisfy the geometric criteria specified below). An additional set of substitutions came from analysis of the subunit-subunit interface in the structural model of FasL derived as described above. These mutations, I168A, L170A, M229A, Y232A, and V248A, correspond to positions distant from the Fas-FasL contact region, but involved in inter-subunit interaction. Other substitutions may be derived from the geometric criteria that the amino acids are at least about 7 Å away from Fas in the model of the Fas-FasL complex, and that at least one of their atoms is within extended van der Waals contact with any atom in a different FasL subunit. ("Extended" van der Waals contact means that the standard van der Waals radii have been increased by the radius of a water molecule; i.e., by 1.4 Å). This additional set includes amino acids T234, R241, S242, L245, G246, A247, F249, S272, Y279, and L281. All these amino acids may be substituted with any of the other 20 standard amino acids, especially with alanine. Other substitutions reducing the ability of FasL to form dimers, trimers, etc. may be found by respectively mutagenizing each amino acid of FasL, for example by replacement with alanine, followed by expression and characterization of the product. Combinations of 2, 3 or more of the substitutions in FasL may be constructed to achieve additive effects.

In another aspect, the invention is directed to recombinant DNA segments that encode all or part of the FasL fusion protein. In the case of an Ig-FasL fusion protein, an exemplary DNA sequence encoding the C<sub>H</sub>3-linker-FasL portion of the molecule is shown in Figure 6. Sequences of the antibody light chain as well as the V<sub>H</sub>, C<sub>H</sub>1, hinge, C<sub>H</sub>2 and C<sub>H</sub>3 domains are well-known (see Sequences of Proteins of Immunologic Interest, 5th ed., E. Kabat et al., U.S. Department of Health and Human Services, 1991,

which is incorporated herein by reference), and are readily available or may be cloned or synthesized in cDNA, genomic, or synthetic form using a variety of methods well-known in the art from, e.g., B cells or hybridomas. The V regions of particular antibodies of interest may be readily cloned using, e.g., anchored PCR (see M. S. Co et al., *J. Immunol.* 148:1149, 1992). Sequences encoding other polypeptide binding proteins have been or may similarly be cloned using art-known methods. Sequences encoding FasL are also known (T. Suda et al., *op. cit.* and T. Takahashi et al., *op. cit.*) and may be obtained or re-cloned using, e.g., polymerase chain reaction (PCR). For use in human patients, the human Fas ligand sequence will preferably be used, but the FasL from other species may also be used, especially for testing.

The DNA segments will typically further include expression control sequences operably linked to the fusion protein coding sequences, including a promoter, ribosome binding site, and polyadenylation and/or transcription termination sites. The fusion protein may be expressed by transfected the DNA segments, generally contained on plasmid vectors, into bacterial, yeast, plant, insect or preferably mammalian cells, using calcium-phosphate, electroporation or other art-known methods. For expression in eucaryotic cells, the promoter and optional enhancer are preferably derived from, e.g., immunoglobulin genes, SV40, retroviruses, cytomegalovirus, elongation factor 1 $\alpha$  (U.S. Patent 5,266,491, which is incorporated by reference) and the like. Preferred host cell lines include CHO cells, COS cells, HeLa cells, NIH 3T3 cells, and various myeloma or hybridoma cell lines including Sp2/0 and NS/0. The plasmid vector will also generally contain a selectable marker such as gpt, neo, hyg or dhfr, and an amp, tet, kan, etc. gene for expression in *E. coli*. A variety of plasmid vectors suitable for expression of heterologous proteins including FasL fusion proteins in a variety of cell types are well-known and readily available in the art. The DNA segments will typically encode a leader sequence at the amino terminus of the fusion protein chain(s) to enable secretion of the fusion protein, e.g., for Ig-FasL the leader sequences naturally associated with the antibody are suitable.

Construction of the DNA segments encoding the fusion protein, their linkage to expression control sequences and insertion into plasmids, and transfection into cells and selection and optional gene amplification of fusion protein-expressing cell lines may all be

performed by a variety of methods well-known in the arts of genetic engineering and cell culture including restriction enzyme digests, ligation, oligonucleotide synthesis and PCR (see, e.g., J. Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, 1989, which is hereby incorporated by reference).

5 Mouse or other antibodies to serve as binding polypeptides have been or may be derived using a variety of methods well-known in the art of immunology and hybridoma technology, including i.p. or footpad immunization in adjuvant and fusion of immune B cells with immortalized myeloma cells (see, e.g., E. Harlow et al., *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988, which is hereby incorporated by reference). Such antibodies can be humanized using published methods including CDR-grafting and framework substitution (see European Patent 0451216 and application 92903551.7, and U.S. Patent No. 5,585,089, each of which is incorporated by reference). Alternatively, human antibodies can be derived directly using trioma methodology (see U.S. Patent 4,634,664 which is incorporated by reference), transgenic animals (e.g., WO 15 93/12227 and U.S. Patent No. 5,569,825 each of which is incorporated by reference), or phage display methods (see WO 91/17271 and WO 92/01047), including chain shuffling (WO 92/20791). Each of the aforementioned references is incorporated herein by reference.

Once a transfectant cell line has been selected that expresses and secretes the FasL fusion protein, it may optionally be adapted to grow in serum-free media (e.g., 20 Hybridoma SFM from Gibco BRL) by passaging in decreasing concentrations of serum, and may be subcloned. The fusion protein may then be purified from (preferably serum-free) media in which the expressing cell line has been grown by standard procedures of the art including filtration, precipitation, protein A affinity chromatography, gel filtration, 25 ion exchange chromatography, electrophoretic methods and the like (see, e.g., *Methods in Enzymology*, vol. 182, *Guide to Protein Purification*, M. P. Deutscher, ed., Academic Press, 1990, which is hereby incorporated by reference). Substantially pure preparations of the fusion protein, i.e., at least 90 to 95% homogeneity and preferably 98 to 99% or higher, are preferred for pharmaceutical uses.

30 For administration to patients, the FasL fusion protein will typically be formulated in a pharmaceutically acceptable carrier. A variety of aqueous carriers can be used, e.g.,

water for injection (WFI), or water buffered with phosphate, citrate, acetate, etc. to a pH typically of 5.0 to 8.0, most often 6.0 to 7.0, and/or containing salts such as sodium chloride, potassium chloride, etc. to make the solution isotonic. The carrier may also contain excipients such as human serum albumin, polysorbate 80, sugars or amino acids 5 to protect the active protein. The concentration of fusion protein in these formulations may vary widely from about 0.01 to 100 mg/ml but will most often be in the range 1 to 10 mg/ml. The formulated FasL fusion protein is particularly suitable for parenteral administration, and may be administered as an intravenous infusion or by subcutaneous, intramuscular or intravenous injection, and may also be administered by injection at the 10 site of disease, e.g., intracranially or into the joints.

In another aspect, the invention is directed to the use of the FasL fusion proteins of the present invention as drugs for treatment of autoimmune disease. The fusion proteins are used to treat a wide variety of autoimmune diseases, such as those listed in Fundamental Immunology, op. cit., but especially those which are organ or tissue-specific 15 and/or which are mediated by T cells. Diseases which are especially suitable for treatment with FasL fusion proteins include rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease (ulcerative colitis and Crohn's disease) and insulin-dependent diabetes (type I diabetes). Other suitable diseases include myasthenia gravis, pemphigus vulgaris, idiopathic thrombocytopenic purpura (ITP), and autoimmune vasculitis. 20 Systemic lupus erythematosus and other non-organ specific autoimmune diseases are also possible. The FasL fusion protein also finds use in the treatment of other types of inflammation, such as due to ischemia and reperfusion (e.g., after myocardial infarction, stroke or hemorrhagic shock), or in inflammatory disorders of the skin, such as psoriasis.

The FasL fusion proteins will also find use as drugs for treatment of cancers, such 25 as leukemias, lymphomas, sarcomas and carcinomas including tumors of the breast, colon, lung, prostate, pancreas and other organs. For such use, the binding polypeptide will bind to a cell surface marker expressed on the cancer cells, usually to a greater extent than on normal cells. Many such tumor-associated cell surface markers are well known in the art. The cancer cells will also express Fas, so that when the FasL fusion 30 protein binds to a cancer cell, the FasL moiety can kill that or neighboring cancer cells by delivering an apoptotic signal through their Fas. For example, the binding polypeptide

may be a humanized ABL 364 antibody which binds to the Lewis y antigen expressed on many tumors including those mentioned above, and the FasL fusion protein may then be used to treat patients with those tumors including those mentioned above. Exemplary humanized ABL 364 antibodies are described in EP 92810633.5, in Co et al., *Cancer Res.* 56:1118, 1996, and U.S Patent 5,562,903 (each of which is incorporated by reference in its entirety and for all purposes). A humanized ABL 364 antibody also referred to as hu-BR55-2 comprises light and heavy chain variable regions that are described in, e.g., Figure 3 of Co et al., 1996, *supra*; Figures 12 and 13 of U.S Patent 5,562,903; and in Table 5 *infra*.

Doses of the drug will typically contain from about 0.01 to about 100 mg FasL fusion protein, but most often from about 0.1 to about 10 mg. The dose chosen will be an amount sufficient to alleviate the disease without causing unacceptable side effects as determined, e.g., in a phase II clinical trial. It may be administered once or multiple times, e.g., 1 to 3 times per day, week or month for one to several days, weeks, months or years, or chronically, depending upon the nature and severity of the disease. The FasL fusion proteins will often be administered in combination with other drugs, for example, corticosteroids, non-steroidal anti-inflammatory drugs, cyclosporin A, or methotrexate, or with thrombolytics (e.g., tPA) in the case of ischemia, according to medical practice and the judgement of the physician. The FasL fusion proteins are particularly suitable for co-administration with humanized antibodies, for example against the IL-2 receptor (see European Patent 0451216) or adhesion molecules (see EP 94903357.5 and WO 94/12215, both of which are incorporated by reference).

To treat a particular autoimmune disease, the binding polypeptide portion of the FasL fusion protein is chosen to preferentially or specifically bind to the cells or tissue under attack in that disease. After treatment, the FasL moiety of the fusion protein will then protrude from the surface of the binding cells and preferably destroy any inflammatory cells, for example cytolytic T cells, that would otherwise infiltrate and damage the affected tissue (see Figure 3). For example, to treat multiple sclerosis, the binding polypeptide would typically bind to the surface of neurons or Schwann cells. Thus, the binding polypeptide may for example be an antibody that binds to myelin basic protein or other components of the myelin sheath, or to a receptor for a neurotransmitter,

or may comprise the binding domain of a neurotrophic factor. To treat rheumatoid arthritis, the binding polypeptide should bind to proteins expressed specifically in the joints, for example on cells of the synovium. To treat type I diabetes, the binding polypeptide can bind to a protein expressed on the membrane of pancreatic beta cells,

5 e.g., to GLUT-2 or to the sulphonylurea receptor (see L. Aguilar-Bryan et al., *Science* 268:423, 1995). To treat inflammatory bowel disease, the FasL fusion protein may bind epithelial cells of the colon by comprising an antibody binding to, e.g., sialyl Lewis a or E-cadherin. For myasthenia gravis, the binding polypeptide is preferably an antibody to the acetylcholine receptor; and for ITP, it is an antibody specifically binding to platelets,

10 e.g., to the gpIIb/IIIa glycoprotein.

Thus, for each cell type under attack in an autoimmune disease, a variety of cell surface markers suitable as targets for binding polypeptides are well known in the art of biology. Preferably, the binding polypeptide of the FasL fusion protein should not substantially down-modulate its target upon binding, which would reduce the number of

15 sites available for the FasL fusion protein to bind and therefore the density of FasL on the cell surface. Also, preferably, the binding polypeptide should not substantially inhibit or stimulate the function of its target, which would interfere with the normal function of the binding cells. However, because of the variety of epitopes on a target protein, it is generally easy, e.g., to generate an antibody that does not inhibit the function of the

20 target, as is well-known in the art. In some cases, it will be sufficient for the FasL fusion protein to bind to neighboring cells of those actually under attack, e.g., to other pancreatic cells instead of or in addition to beta cells in treating diabetes. Alternatively, the FasL fusion protein may bind to non-cellular components of the tissues under attack, for example to protein components of basement membranes, or to collagen in the case of

25 rheumatoid arthritis.

To assay the FasL fusion protein in vitro, increasing concentrations of the fusion protein (e.g. from 0.001 ng/ml to 1000 ng/ml) are incubated with Fas expressing cells, e.g. W4 transfectant cells or T cells or neutrophils, and lysis of the Fas expressing cells measured, e.g., by a <sup>51</sup>Cr release assay. See, generally, T. Suda et al., *op. cit.* and T.

30 Suda and S. Nagata, *op. cit.*, which are incorporated herein by reference. As described above, the FasL fusion protein will have reduced ability to cause apoptosis of the cells

relative to the FasL protein or extracellular domain alone. In a second experiment, the binding cells for the FasL fusion protein are added to the incubation mix. As described above, the fusion protein will have greater effectiveness in causing the death of the target cells in the presence of the binding cells. For example, as a test case, the binding 5 polypeptide of the fusion protein is the humanized ABL 364 antibody against the Lewis y antigen and the binding cells are SKBR5 or T47D breast carcinoma cells. In additional experiments, other Fas expressing cells are used, for example T cells activated, e.g., by growth in Con A and IL-2, followed by stimulation with PMA and ionomycin.

As an optional second set of in vitro assays, Fas-expressing cells are incubated 10 with binding cells for which they have lytic activity. For example, the binding cells may be K562 or YAC cells or other cells susceptible to lysis by NK cells, and the Fas-expressing cells may be NK cells activated by, e.g., IL-2 and IL-12. Alternatively, the Fas-expressing cells may be T cells activated as above, or cytotoxic T lymphocyte (CTL) cell lines with specificity for the binding cells, which may be, e.g., tumor cell lines or 15 transfected cells. Methods to generate such specific CTL lines are well-known in the art of immunology. The ability of the Fas-expressing cells to lyse the binding cells is assayed by art-known techniques, such as <sup>51</sup>Cr labeling of the binding cells. This ability is measured in the absence and presence of various concentrations of a FasL fusion protein which comprises a binding polypeptide that binds to the binding cells. Presence 20 of the FasL fusion protein inhibits the ability of the Fas-expressing cells to lyse the binding cells, because the FasL fusion protein causes the apoptosis of the "attacking" Fas-expressing cells after it binds to the binding cells, thus protecting them (Figure 3). This in vitro experiment therefore models the use of FasL fusion proteins to treat autoimmune disease, in which the FasL fusion proteins protect the cells under autoimmune attack by 25 binding to them and apoptosis of infiltrating inflammatory cells, as described above.

The FasL fusion proteins can also be assayed in a variety of in vivo animal models. For example, to establish their ability to successfully treat multiple sclerosis, they may be assayed in mouse or rat experimental allergic encephalomyelitis (EAE). To establish their ability to treat rheumatoid arthritis, they may be assayed in collagen- or 30 adjuvant-induced arthritis in rats. Reduction of disease severity in these models is measured by scales that respectively indicate degree of paralysis or joint swelling, as

commonly used in the art. Survival after a defined time may also be an appropriate endpoint in certain animal models. Ability to treat diabetes can be modeled in the non-obese diabetic (NOD) mouse or BB rat. A large number of animal models are available to test the treatment of these and other autoimmune diseases (see, e.g., European Patent 5 0304291 and references cited therein) or other inflammatory diseases (see EP 94903357.5 and references cited therein) including inflammatory bowel disease (see C. Elson et al., *Gastroenterology* 109:1344, 1995). Of course, it is understood that the binding polypeptide of the FasL fusion protein will bind to the appropriate cell type in the animal species used. The ability of FasL fusion proteins to treat cancer is shown by their ability 10 to prevent, inhibit or reverse growth of murine or human tumors in normal or immunocompromised mice.

In the case of each animal model, before, at or after induction of the disease (e.g., 1, 3, 5, 7, 9 or 10 - 14 or more days after), at least one dose of the appropriate FasL fusion protein is administered, typically i.p. but possibly i.v. or by another route. 15 Multiple doses of the FasL fusion protein may be administered on these or other days. The size of the dose will be scaled from the typical doses described above for human patients, but may be somewhat larger proportionally, i.e., will vary from approximately 1 ng to 1 mg per animal, but most often will be from 1 to 100  $\mu$ g. The severity of the disease will then be measured on various days after treatment, and compared with 20 untreated or placebo-treated animals. Treatment with the FasL fusion protein will reduce the appropriately measured severity of the disease and may decrease animal mortality.

The FasL fusion proteins will also find other uses. For example, they may be used to prevent or treat rejection of organ transplants, including xenotransplants, by choosing as the binding polypeptide component an antibody that binds specifically to the 25 transplanted organ, e.g., to MHC determinants expressed on it. They may be used in vitro to detect or to lyse Fas-expressing cells in a mixed population of Fas- and non-Fas-expressing cells. They may serve as the detecting reagent in a diagnostic assay, e.g., ELISA assay, for soluble Fas or Fas-expressing cells in patients with leukemia or other disorders.

EXPERIMENTALExample 1: Cloning of FasL cDNA

A cDNA clone of the human FasL gene is obtained, or cloned again by first synthesizing single strand cDNA from RNA extracted from human FasL expressing cells 5 such as human peripheral blood lymphocytes or activated T lymphocytes, using oligo dT as a primer. Then the cDNA is used as a template for PCR with primers (1) and (2) of Table 1 below, or other primers based on the sequence of the human FasL gene (T. Tabahashi et al., *Internat. Immunol.* 6:1567, 1994, which is incorporated herein by reference).

10

Table 1. Primers used in construction of Ig-FasL fusion protein

	Number	Sequence
	1	ATGCATGCTCTAGAATGCAGCAGCCCTCAATTACCC [Seq. ID No. 3]
15	2	ATGCATGCTCTAGATTAGAGCTTATATAAGCCG [Seq. ID No. 4]
	3	ACCACAGGTGTACACCCTGC [Seq. ID No. 5]
	4	ATGCATGCGGTACCTTACTCGGAGACAGGGAGAGG [Seq. ID No. 6].
20	5	ATGCATGCGGTACCTGAGTGCCACGGCCGGCAAG [Seq. ID No. 7]
	6	GGGAAGTATGTACACGGGG [Seq. ID No. 8]
	7	AGCAAATAGGATCCCCAGTCC [Seq. ID No. 9]
	8	ATGCATGCGGTACCTTAGAGCTTATATAAGCCG [Seq. ID No. 10]
25	9	ATGCATGCGGTACCCAGCTCTCCACCTACAGAAG [Seq. ID No. 11]
	10	GGACTGGGGATCCTATTGCTTCTCCAAAG [Seq. ID No. 12]

Example 2: Construction of Ig-FasL in expression vector

In this example, an Ig-FasL fusion protein is made that incorporates an antibody of the human IgG2 isotype, utilizing the pVg2 expression vector (Figure 4), which is the same as pVg1 (Co et al., *op cit*), except that the XbaI - BamHI segment containing the  $\gamma_1$  constant region has been replaced using standard methods with a genomic segment containing the  $\gamma_2$  constant region. First, a Kpn I site (GGTACC) is introduced between the last coding codon and the termination codon of the IgG2 C<sub>H</sub>3 domain in pVg2 by PCR as follows. Using the IgG2 C<sub>H</sub> gene as template, PCR with primers (3) and (4) of Table 1 above generates a 321 bp fragment that extends from a BsrGI site at the fourth codon through the end of the C<sub>H</sub>3 coding region. PCR with primers (5) and (6) generates a 101 bp fragment that contains the sequence from the end of the C<sub>H</sub>3 coding region through the BsrGI site about 80 bp downstream. Both of the PCR product fragments are digested with KpnI and BsrGI, joined at the Kpn I ends, and used to replace the corresponding BsrGI fragment in pVg2.

A fusion of the human IgG2 C<sub>H</sub> gene and part of the human FasL gene is then constructed in the following manner. A 453 bp BamHI - KpnI DNA fragment encoding the C-terminal extracellular 145 amino acids of human FasL is generated by PCR using the human FasL cDNA clone as template and primers (7) and (8) followed by digestion with KpnI and BamHI. A 93 bp KpnI - BamHI DNA fragment encoding a polypeptide linker is generated by PCR also using the human FasL cDNA clone as template but with primers (9) and (10), again followed by digestion with Kpn I and Bam HI. These two fragments are joined at the Bam HI ends and inserted into the KpnI site at the end of the CH3 coding region of the modified pVg2 plasmid. The resulting encoded fusion protein, which is contained on the pVg2FasL plasmid (Figure 5), consists of a complete human IgG2 C<sub>H</sub> region sequence, followed by Gly and Thr amino acids due to the introduction of the Kpn I site, a polypeptide linker consisting of the membrane domain-proximal 34 amino acids of the extracellular domain of human FasL (amino acids 103 through 136) with a His to Ser substitution at the 31st position, and the 145 C-terminal (extracellular) residues of human FasL. Of course, any desired polypeptide linker, e.g., amino acids 132 through 136 of FasL, can be used instead of the one chosen here by encoding it on a KpnI - BamHI fragment by oligonucleotide synthesis, and using that fragment instead of

the 93 bp KpnI - Bam HI DNA fragment described above. Similarly, an analogous construction starting from pVg1 or pVg4 (see EP 94903357.5) or analogously constructed pVg3 vector can be used to generate FasL fusion proteins using antibodies respectively of the IgG1, IgG4 and IgG3 isotypes.

5

Example 3: Expression of Ig-FasL protein

To express an Ig-FasL protein binding to a particular epitope, the variable regions of an antibody with that specificity are cloned, and the  $V_H$  gene including signal sequence and splice donor sequence inserted at the XbaI site of pVg2FasL. The  $V_L$  gene of the 10 antibody is similarly cloned into the XbaI site of the light chain expression vector pV<sub>k</sub> (Co et al., 1992, *op cit*), and the two expression plasmids co-transfected into an appropriate cell line, such as Sp2/0 cells, by electroporation. Cells are selected for gpt expression and screened for production of IgG2-FasL fusion protein by ELISA using an 15 anti-human light chain capture reagent and an anti-human heavy chain or anti-human FasL detection reagent. The IgG2-FasL fusion protein is purified from culture supernatant of a high-yielding transfected cell line by protein A affinity chromatography or other chromatographic techniques. As one example, the light and heavy chain genes of the humanized ABL 364 antibody are inserted into the vectors as indicated to express a fusion protein of humanized ABL 364 and FasL.

20

Example 4: Additional Ig-FasL fusion protein constructs

Other expression plasmids encoding Ig-FasL fusion proteins were constructed. As in Example 2, a KpnI site was first introduced between the last coding codon and the termination codon of the human IgG2 heavy chain constant region gene segment in vector 25 pVg2 (Fig. 4). Specifically, using pVg2 as template, PCR with the appropriate primers generated a BsrGI-KpnI fragment product extending from the BsrGI site at the fourth codon of the CH3 domain to the end of the CH3 coding region. A second PCR using the same template and other appropriate primers generated a KpnI-BsrGI fragment product 30 extending from the end of the CH3 coding region to the BsrGI site approximately 80 bp downstream. These two PCR products were joined at the KpnI site ends and used to

replace the corresponding BsrGI fragment of pVg2. The resulting plasmid was designated pVg2Kpn.

Also similarly to Example 2, KpnI fragments encoding the extracellular portion of human FasL preceded by a short FasL linker region of either 5 or 34 amino acid residues 5 were generated by PCR with the appropriate primers and using the human FasL cDNA clone as template. Each of these fragments was inserted into the KpnI site of pVg2Kpn to generate plasmids encoding fusion polypeptides consisting of a human IgG2 heavy chain constant region followed by a Gly Thr dipeptide due to the introduction of the KpnI site, a 5 or 34 residue FasL linker, and the extracellular human FasL domain. These 10 plasmids were designated pVg2hFasL5 and pVg2hFasL34.

Complete heavy chain-FasL genes were constructed by inserting an XbaI fragment encoding the heavy chain variable region of the humanized ABL 364 antibody denoted HuABL364 (M. S. Co et al., *Cancer Res.* 56: 1118, 1996, incorporated herein by reference) into the XbaI site upstream of the heavy chain constant region coding region in 15 plasmids pVg2hFasL5 and pVg2hFasL34. The resulting plasmids were designated pABLhFasL5 and pABLhFasL34 respectively.

To provide a selectable marker on the pABLhFasL5 and pABLhFasL34 plasmids, the 1.5 kb HindIII-BamHI fragment containing the dhfr gene was replaced with the 2.3 kb Hind III-Bam HI fragment from pSV2neo (P. J. Southern and P. Berg, *J. Mol. Appl. 20 Genet.*, 1:327, 1982) that encodes the gene for neomycin resistance. The resulting plasmids were designated pABLhFasL5neo and ABLhFasL34neo.

#### Example 5: Expression of HuABL364 Ig-FasL fusion proteins

Humanized ABL 364 Ig-FasL fusion proteins were expressed in both stably and 25 transiently transfected cells. NIH 3T3 cells were stably co-transfected with the plasmids pABLhFasL5neo or pABLhFasL34neo together with the HuABL364 light chain expression plasmid phABLTEWL (M. S. Co et al., *Cancer Res.* 56: 1118, 1996) using the liposome reagent Lipofectamine (GibcoBRL) following the manufacturer's protocol. Transfected cells were selected for resistance to the drug G418.

30 COS7 cells were transiently co-transfected with phABLTEWL and either pABLhFasL5 or pABLhFasL5neo, or pABLhFasL34 or pABLhFasL34neo, using

Lipofectamine (GibcoBRL) following the manufacturer's protocol. Ig-FasL protein produced by the former plasmids contains the 5 amino acid linker between the Ig and FasL and is denoted HuABL364 Ig-FasL5, whereas Ig-FasL protein produced by the latter plasmids contains the 34 amino acid linker and is denoted HuABL364 Ig-FasL34; 5 the two forms are generically denoted HuABL364 Ig-FasL.

Production of HuABL364 Ig-FasL protein by stable and transient transfectants was demonstrated by ELISA and immunoprecipitation. ELISA analysis utilized either of two capture reagents, a polyclonal goat anti-human gamma chain reagent (Biosource, #AHI1301) and a mouse monoclonal anti-human FasL antibody (Pharmingen, #65321A). 10 The developing reagent was a peroxidase-conjugated goat anti-human kappa chain antibody (Biosource, #AHI0804 or Southern Biotech, #2060-05). In an ELISA with either capture reagent, the stably transfected cells gave a positive signal above background, showing that the secreted protein contained both the human gamma chain and FasL components.

15 HuABL364 Ig-FasL protein was immunoprecipitated from culture supernatents of transiently transfected cells using either protein A Sepharose 4B (Sigma) or goat anti-human IgG agarose (Sigma) following art-known procedures. Polyacrylamide gel electrophoresis (PAGE) under denaturing and reducing conditions of the immunoprecipitated protein yielded bands of the expected size for HuABL364 Ig-FasL 20 upon staining with Coomassie Blue. HuABL364 Ig-FasL was purified from culture supernatents of transiently or stably transfected cells by affinity chromatography on protein G columns.

Example 6: Characterization of HuABL364 Ig-FasL

25 The ability of HuABL364 Ig-FasL to bind Fas on the cell surface via the FasL domain was demonstrated by flow cytometry. MOLT-4, a human T lymphocyte line that expresses Fas on the cell surface (B. Trauth et al., *Science*, 245:301, 1989, incorporated herein by reference) but not the antigen for HuABL364, was stained by incubation with protein G-purified HuABL364 Ig-FasL followed by incubation with an anti-human IgG 30 FITC reagent (Jackson Immunoresearch, #715-096-151). Positive staining by HuABL364 Ig-FasL was detected by flow cytometry, compared to negative staining with the

HuABL364 antibody. Moreover, an anti-Fas mouse monoclonal antibody (Calbiochem, fas Ab-2) inhibited staining by HuABL364 Ig-FasL, demonstrating that binding of HuABL364 Ig-FasL to MOLT-4 cells was due to FasL-Fas interaction, and therefore that FasL was functional in the Ig-FasL construct.

5 The ability of protein G-purified HuABL364 Ig-FasL to kill cells by apoptosis was determined by flow cytometry using a commercially available assay based on cell staining by annexin V and propidium iodine, following the manufacturers protocol (R&D Systems, Minneapolis, MN; cat. # KNX50). HuABL364 Ig-FasL, both soluble and bound to a solid phase (i.e., plastic surface), demonstrated apoptotic activity toward CESS cells

10 (Table 2), a human B lymphocyte that expresses Fas (B. Trauth et al., *Science*, 245:301, 1989, incorporated herein by reference).

Table 2. Apoptotic activity toward CESS cells of HuABL364 Ig-FasL

	<u>Protein</u>	<u>Phase</u>	<u>% apoptotic cells</u>
15	HuABL364 Ig-FasL34	solution	61.0%
	HuABL364 Ig-FasL34	solid phase	44.6%
	Anti-Fas IgG antibody	solution	15.3%
	Anti-Fas IgG antibody	solid phase	41.3%
	No protein		11.8%

20

To determine whether the apoptotic activity of the soluble HuABL364 Ig-FasL is due to oligomerization of the protein, a sample was analyzed using an HPLC gel filtration column (Tosohhaas G3000 SW). A substantial amount of the Ig-FasL eluted at a position corresponding to a molecular weight of about 600,000 Daltons, suggesting that aggregates 25 of the protein are formed. This is likely to be a result of interaction between FasL domains of multiple Ig-FasL molecules.

Example 7: Construction and analysis of Ig-FasL mutant fusion proteins

KpnI fragments encoding the nine FasL mutants H148S, Y189A, Y192A, Y244A, 30 I168A, L170A, M229A, Y232A, and V248A described above were generated by PCR using the appropriate primers and pABLhFasL5neo as template. The KpnI fragment for

each mutant was inserted into pABLhFasL5neo, replacing the wild type fragment, resulting in expression plasmids encoding the heavy chain - FasL mutant polypeptides.

The mutant and wild type HuABL364 Ig-FasL were expressed by transient transfection of COS7 cells as described above. The presence of Ig-FasL protein in the culture supernatents of transiently transfected cells was verified by ELISA using the anti-human gamma and anti-FasL capture reagents as described above. The culture supernatants of the transfected cells containing the respective HuABL364 Ig-FasL proteins were used for further experiments, with supernatant from untransfected cells serving as a negative control.

10 The apoptosis-inducing activity of the wild type and mutant Ig-FasL proteins (culture supernatant from transfected cells) was tested, as described above, on two target cell lines: the CESS line described above which expresses Fas but not the ABL 364 antigen, and T47D, a human breast carcinoma cell that expresses both Fas and the ABL 364 antigen. The apoptotic activity of the Ig-FasL proteins fall into three classes (Table 15 3). One class induces apoptosis in both of the cell lines (wild type, I168A, L170A, M229A, and V248A). A second class is unable to induce apoptosis in either cell line (H148S, Y189A, Y192A, and Y244A). And a third class has activity against T47D cells but not CESS cells (Y232A). The third class is the most preferred in that the Ig-FasL fusion protein has little or no apoptotic activity when in solution, but when cross-linked 20 or aggregated by binding to cells expressing the ABL 364 antigen, demonstrates apoptotic activity. Moreover, such fusion proteins have reduced ability in vitro, relative to soluble FasL protein or FasL protein extracellular domain, to cause death of cells expressing Fas protein.

Ig-FasL fusion proteins (e.g., comprising the humanized ABL 364 variable 25 domain) that have an amino acid substitution at Y232, especially of alanine, will therefore have the ability to cause death in a first population of cells expressing Fas protein when such first population of cells are in the presence of a second population of cells to which the polypeptide binds (e.g., cancer cells that express the Lewis y antigen), increased relative to the absence of such second population of cells.

Table 3. Apoptotic activity of HABL364 Ig-FasL mutant fusion proteins

		Mutation in	<u>% apoptotic cells</u>	
		<u>HuABL364 Ig-FasL5</u>	<u>CESS</u>	<u>T47D</u>
5		No mutation (wild-type)	88%	91%
		H148S	26%	15%
		I168A	81%	91%
		L170A	87%	98%
10	Y189A		24%	11%
	Y192A		25%	10%
	M229A		90%	95%
	Y232A		32%	88%
	Y244A		24%	16%
15	V248A		87%	95%
	Untransfected			
	supernatant		27%	14%

20 From the foregoing, it will be appreciated that the FasL fusion proteins of the present invention offer numerous advantages over other treatments for autoimmune disease or cancer. Individual FasL fusion proteins are applicable to many different autoimmune or other inflammatory conditions or cancers, are efficacious, and because they target only the organs and cells involved in a particular disease, have few side effects. They may be readily and economically produced, require only relatively small doses, and generally have little or no immunogenicity.

25 All publications and patent filings are herein incorporated by reference to the same extent as if each individual publication or patent filing was specifically and individually indicated to be incorporated by reference. Although the present invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be apparent that certain changes and modifications may be practiced within the scope of the appended claims.

26  
Table 4

Amino Acid Sequence [Seq ID No 2] of FasL,  
and FasL Nucleotide Sequence [Seq. ID. No. 1]

1	ATG	CAG	CAG	CCC	TTC	AAT	TAC	CCA	TAT	CCC	CAG	TAC	TCC	GTC	GAC	AGC	GCC	AGC		
1	►Met	Gln	Gln	Pro	Pho	Asn	Tyr	Pro	Tyr	Pro	Gln	118	Tyr	Tyr	Val	Asp	Ser	Ser	Ala	Ser
61	TCT	CCC	TGG	GCC	CCT	CCA	GCA	GTC	CTT	CCC	TGT	CTT	GTC	CCC	AGA	AGG	CCT			
21	►Ser	Pro	Tyr	Ala	Pro	Pro	Gly	Thr	Val	Leu	Pro	Cys	Pro	Thr	Ser	Val	Pro	Arg	Arg	
121	GGT	CAA	AGG	CCA	CCA	CCA	CCA	CCG	CCA	CCA	CCA	CCT	CCT	CCG	CCG	CCG	CCG			
41	►Gly	Gln	Arg	Pro	Leu	Pro	Pro	Pro	Pro											
181	CCA	CCA	CTG	CCT	CCA	CTA	CCG	CTG	CCA	CCC	CTG	AAG	AAG	AGA	GGG	AAC	CAC	AGC		
61	►Pro	Pro	Leu	Pro	Pro	Leu	Pro	Pro	Pro	Pro										
241	CTG	TGT	CTC	CTT	GTG	ATG	TTT	TTC	ATG	GTC	GTG	TTT	GCC	TTG	GTA	GGA	TTG	GGC	CTG	
81	►Leu	Cys	Leu	Leu	Val	Met	Val	Leu	Val	Ala	Leu	Val	Gly	Leu	Leu	Gly	Leu	Gly		
301	ATG	TTT	CAG	CTC	TTC	CAC	CTA	CAG	AAG	GAG	CTG	GCA	GAA	CTC	CGA	GAG	TCT	ACC	AGC	
101	►Met	Pho	Gln	Leu	Pho	His	Leu	Gln	Lys	Glu	Leu	Ala	Glu	Leu	Arg	Glu	Ser	Thr	Gln	
361	ATG	CAC	ACA	GCA	TCA	TCT	TTG	GAG	AAG	CAA	ATA	GCC	CAC	CCC	AGT	CCA	CCC	CCT	GAA	
121	►Met	His	Thr	Ala	Ser	Ser	Leu	Glu	Lys	Gln	118	Gly	His	Pro	Ser	Pro	Pro	Pro	Gly	
421	AAG	GAG	CTG	AGG	AAA	GTG	GCC	CAT	TTA	ACA	GCC	AAG	TCC	AAC	TCA	AGG	TCC	ATG	CTG	
141	►Lys	Glu	Leu	Arg	Lys	Val	Ala	His	Leu	Thr	Gly	Lys	Ser	Asn	Ser	Arg	Ser	Met	Pro	
481	GAA	TGG	GAA	GAC	ACC	TAT	GGA	ATT	GTC	CTG	CTT	GGA	GTC	AAG	TAT	AAG	AAG	GGT	GCC	
161	►Glu	Tyr	Asp	Thr	Tyr	Gly	118	Val	Leu	Ser	Gly	Val	Leu	Tyr	Lys	Lys	Gly	Gly		
541	CTT	GTG	ATC	AAT	GAA	ACT	GGG	CTG	TAC	TTT	GTA	TAT	TCC	AAA	GTA	TAC	TTC	CGG	CAA	
181	►Leu	Val	118	Asn	Glu	Thr	Gly	Leu	Tyr	Pho	Val	Tyr	Ser	Lys	Val	Tyr	Pho	Arg	Gly	
601	TCT	TGC	AAC	AAC	CTG	CCC	CTG	AGC	CAC	AAG	GTC	TAC	ATG	AGG	AAC	TCT	AAG	TAT	CCC	
201	►Ser	Cys	Asn	Asn	Leu	Pro	Leu	Ser	His	Lys	Val	Tyr	Met	Arg	Asn	Ser	Lys	Tyr	Pro	
661	GAT	CTG	GTC	ATG	ATG	GAG	GGG	AAG	ATG	AGC	TAC	TGC	ACT	ACT	GGG	CAG	ATG	TGG	GCC	
221	►Asp	Leu	Val	Met	Met	Gly	Lys	Met	Met	Ser	Tyr	Cys	Thr	Thr	Gly	Gln	Met	Trp	Ala	
721	CCG	AGC	TAC	CTG	GGG	GCA	GTG	TTC	AAT	CTT	ACC	AGT	GCT	GAT	CAT	TTA	TAT	GTC	AAC	
241	►Arg	Ser	Ser	Tyr	Leu	Gly	Ala	Val	Pho	Asn	Leu	Thr	Ser	Ala	Asp	His	Leu	Tyr	Val	
781	GTA	TCT	GAG	CTC	TCT	GTC	AAT	TTT	GAG	GAA	TCT	CAG	ACG	TTT	TTC	GGC	TTA	TAT	AAG	
261	►Val	Ser	Glu	Leu	Ser	Leu	Val	Asn	Pho	Glu	Leu	Ser	Gln	Thr	Pho	Pho	Gly	Leu	Tyr	
841	CTC	TAA																		
281	►Leu	...																		

Table 5

Amino Acid Sequence of the Mature Heavy Chain (A) [Seg. ID No. 13] and Light Chain (B)  
[Seq. ID No. 14] Variable Regions of Humanized ABL 364 Antibody

(A)

1	E V Q L L E S G G G L V Q P G G S L R L
21	S C A A S G F T F S D Y Y M Y W V R Q A
41	P E K R L E W V A Y I S N G G G S S H Y
61	V D S V K G R F T I S R D N A K N T L Y
81	L Q M N S L R A E D T A L Y H C A R G M
101	D Y G A W F A Y W G Q G T L V T V S S

(B)

1	D I V M T Q S P L S L P V T P G E P A S
21	I S C R S S Q S I V H S N G N T Y L E W
41	Y L Q K P G Q S P Q L L I S K V S N R F
61	S G V P D R F S G S G S G T D F T L K I
81	S R V E A E D V G V Y Y C F Q G S H V P
101	F T F G Q G T K L E I K

CLAIMS

We claim:

- 1        1. A fusion protein comprising a recognition moiety of the extracellular domain of a Fas ligand protein and a polypeptide capable of specifically binding to a cell surface marker.
- 1        2. A fusion protein of claim 1 further comprising a polypeptide linker between said extracellular domain of the Fas ligand protein and said polypeptide capable of specifically binding the cell surface marker.
- 1        3. A fusion protein of claim 2 wherein said linker is between 8 and 40 amino acids in length.
- 1        4. A fusion protein of claim 3 wherein said linker is substantially identical to a sequence occurring in a natural human protein.
- 1        5. A fusion protein of claim 1 wherein said binding polypeptide comprises the variable domain of an antibody.
- 1        6. A fusion protein of claim 5 wherein said binding polypeptide is an antibody.
- 1        7. A fusion protein of claim 6 wherein said antibody is humanized or human.
- 1        8. A fusion protein of claim 7 wherein said antibody is of the human IgG2 or IgG4 isotypes.

1           9.    A fusion protein of claim 1 that has reduced ability *in vitro*, relative to  
2 soluble FasL protein or FasL protein extracellular domain, to cause death of cells  
3 expressing Fas protein.

1           10.   A fusion protein of claim 1 that has increased ability *in vitro* to cause death  
2 in a first population of cells expressing Fas protein, when such first population of cells  
3 are in the presence of a second population of cells to which the polypeptide binds, relative  
4 to the absence of such second population of cells.

1           11.   A fusion protein of claim 1 wherein said polypeptide specifically binds to  
2 neurons, pancreatic beta cells, synovial cells, or colonic epithelial cells.

1           12.   A DNA segment encoding the fusion protein of claim 1.

1           13.   A cell line producing the fusion protein of claim 1.

1           14.   A fusion protein of claim 1 which is substantially pure.

1           15.   A pharmaceutical composition comprising a fusion protein of claim 1 in a  
2 pharmaceutically acceptable carrier.

1           16.   A method of treating an autoimmune disease or cancer comprising  
2 administering to a patient in need of such treatment one or more doses of the fusion  
3 protein of claim 1.

1           17.   The method of claim 16, wherein said disease is multiple sclerosis,  
2 rheumatoid arthritis, insulin-dependent diabetes, or inflammatory bowel disease.

1           18.   A method of preventing or treating a disease comprising administering to a  
2 patient a fusion protein comprising a recognition moiety of the Fas ligand protein and a  
3 polypeptide that preferentially binds to the tissues affected by said disease.

1           19. The method of claim 18, wherein said disease is multiple sclerosis,  
2           rheumatoid arthritis, type I diabetes, inflammatory bowel disease, psoriasis, or rejection  
3           of an organ transplant.

1           20. The method of claim 18, wherein said disease is cancer of the breast, lung,  
2           colon, ovary or prostate.

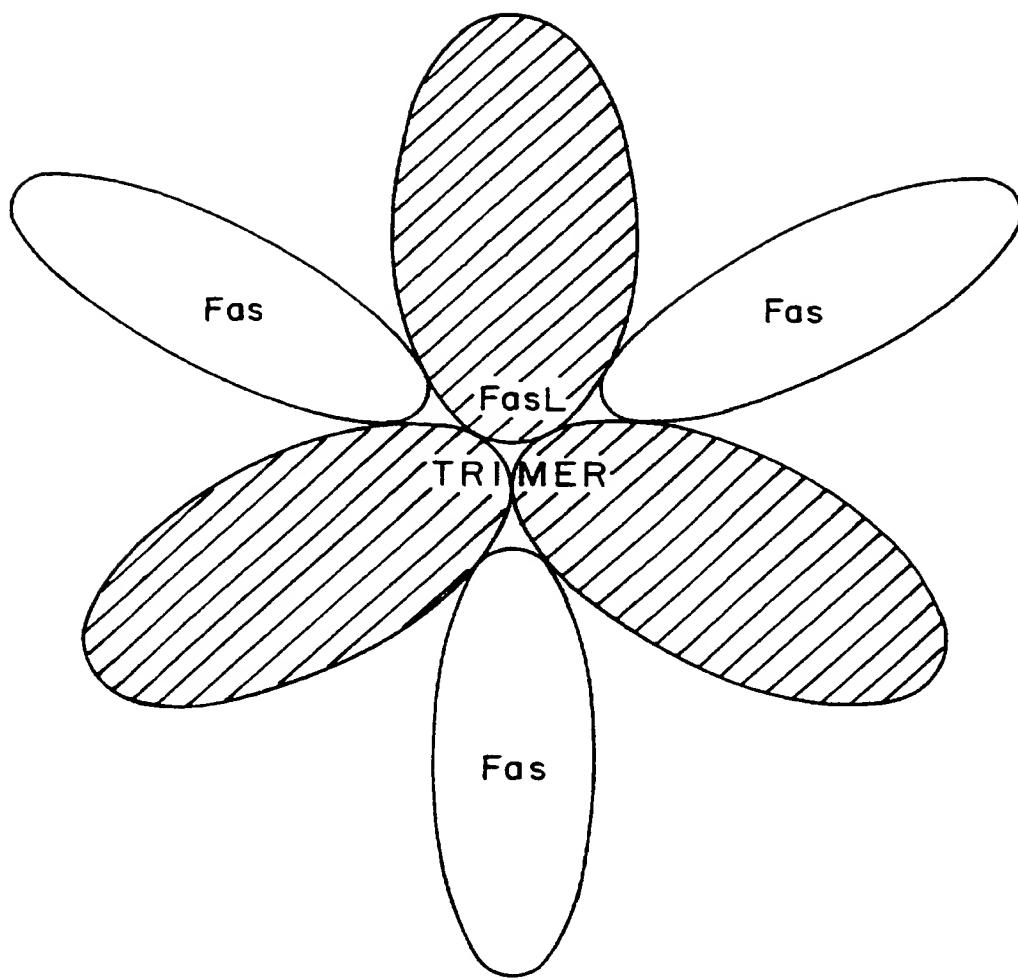
1           21. A fusion protein of claim 1, 2, 9 or 10 comprising an amino acid  
2           substitution in said Fas ligand protein that decreases the ability of the fusion protein to  
3           form dimers, trimers or other aggregates.

1           22. A fusion protein of claim 21 wherein said substitution occurs at amino acid  
2           148, 189, 192, 244, 168, 170, 229, 232, 248, 234, 241, 242, 245, 246, 247, 249, 272,  
3           279 or 281, as numbered in Table 5.

1           23. A fusion protein of claim 22 wherein said substitution occurs at the amino  
2           acid 232.

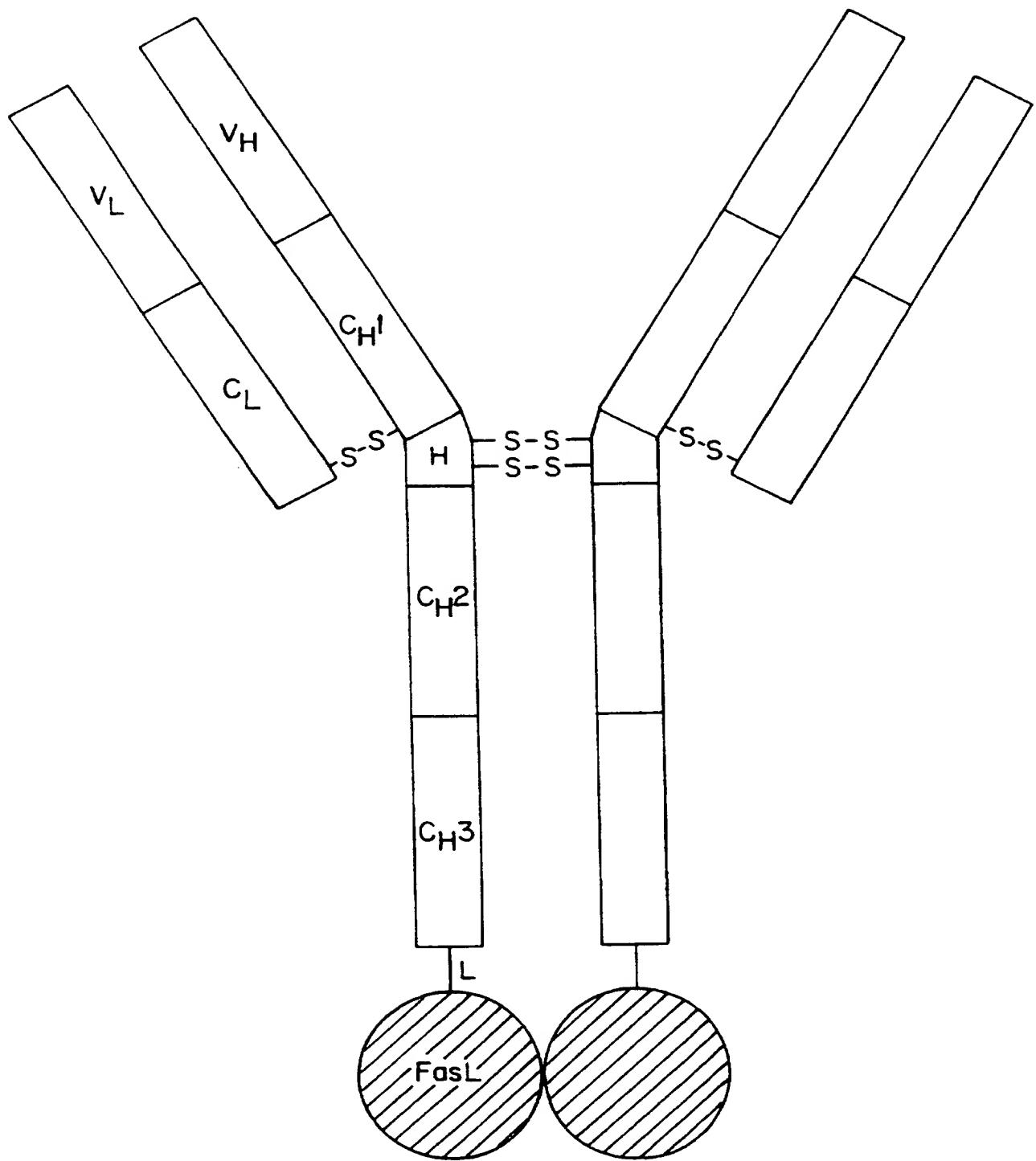
1           24. A fusion protein of claim 23 wherein the amino acid 232 is alanine.

1           25. A fusion protein of claim 21, wherein the binding polypeptide comprises  
2           the heavy and light chain variable regions of a humanized ABL 364 antibody.

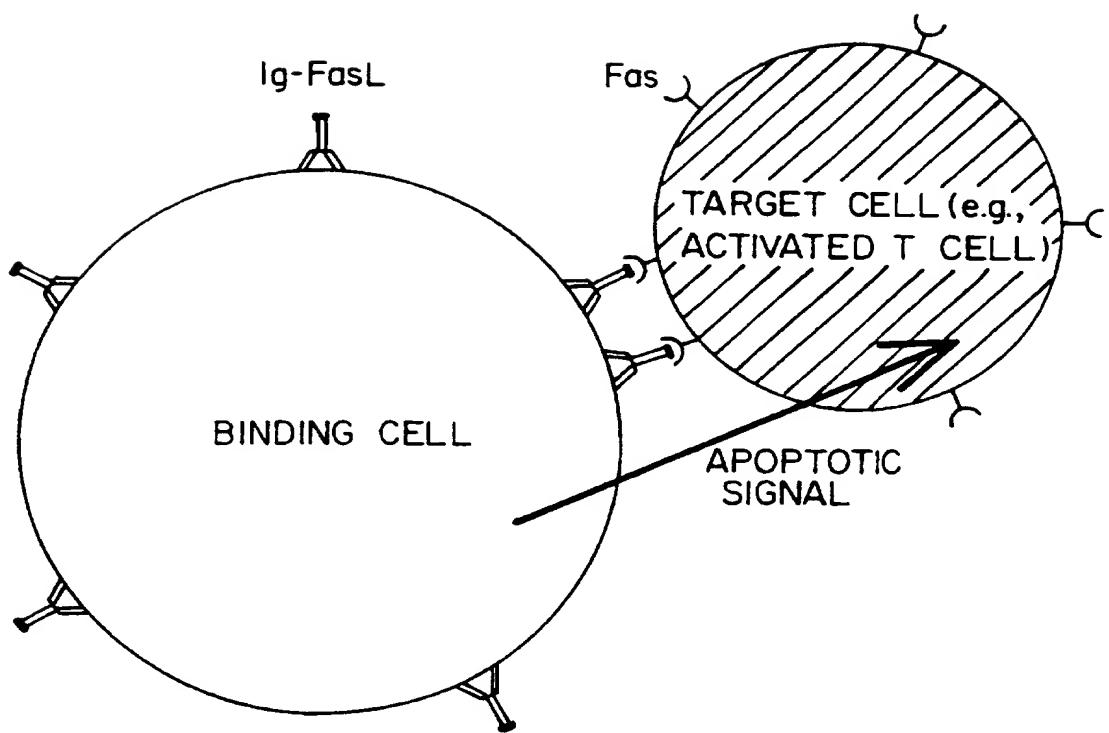


*FIG. 1.*

**SUBSTITUTE SHEET (RULE 26)**

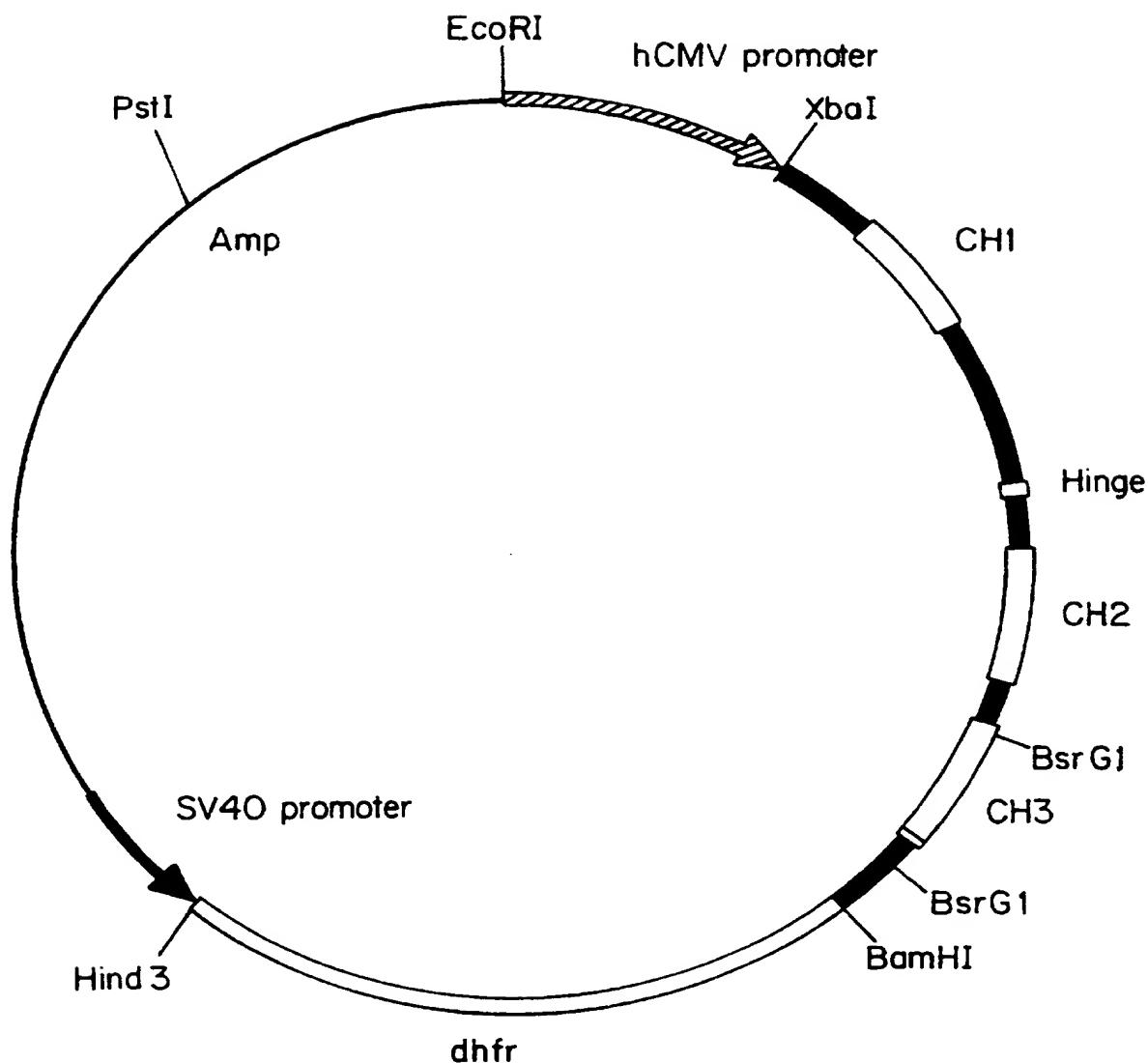


**FIG. 2.**  
SUBSTITUTE SHEET (RULE 26)

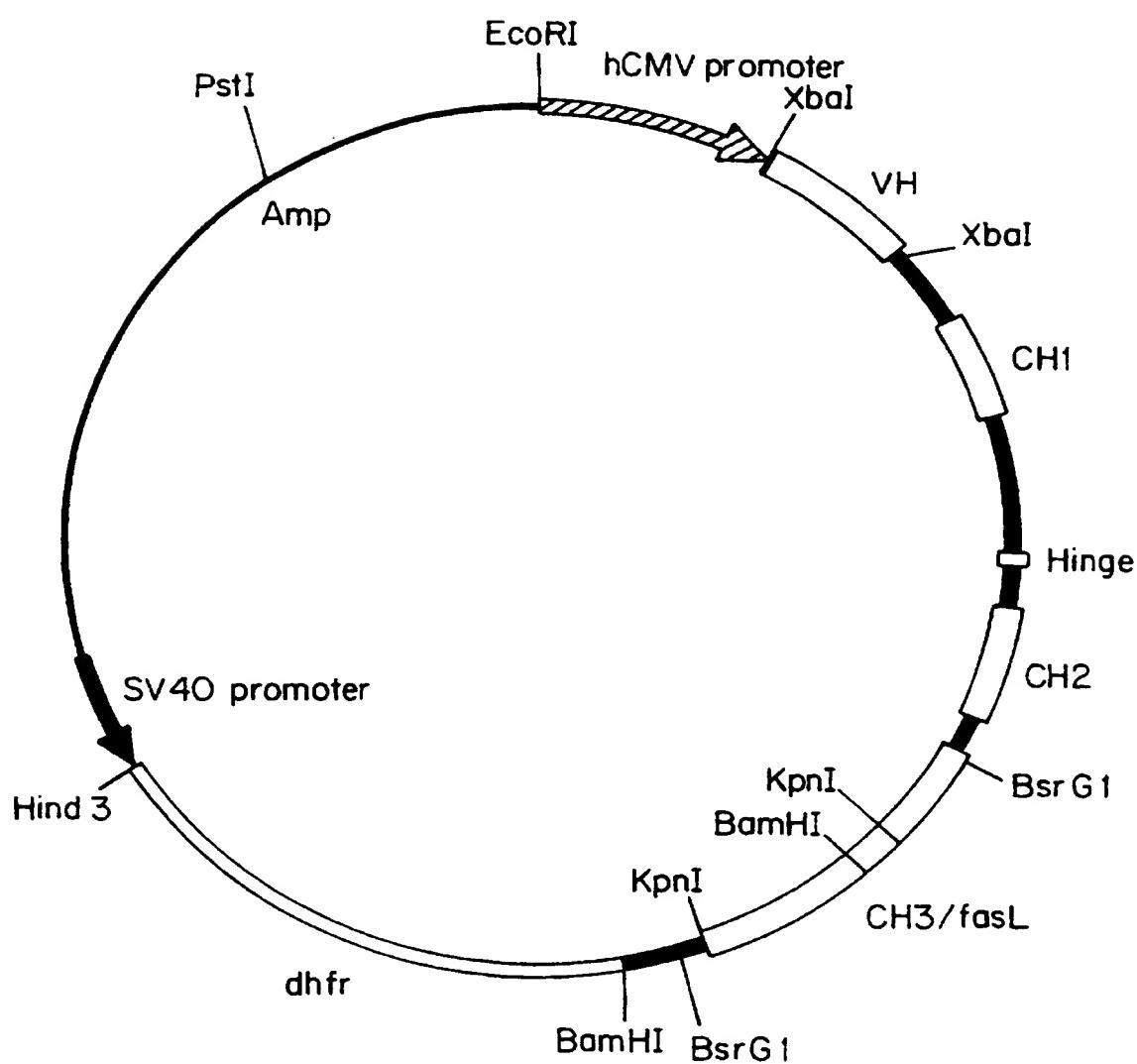


*FIG. 3.*

**SUBSTITUTE SHEET (RULE 26)**



**FIG. 4.**



*FIG. 5*

SUBSTITUTE SHEET (RULE 26)

6/7

1 CAG CCC CGA GAA CCA CAG GTG TAC ACC CTG CCC CCA TCC  
1 Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser

40 CGG GAG GAG ATG ACC AAG AAC CAG GTC AGC CTG ACC TGC  
14 Arg Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys

79 CTG GTC AAA GGC TTC TAC CCC AGC GAC ATC GCC GTG GAG  
27 Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu

118 TGG GAG AGC AAT GGG CAG CCG GAG AAC AAC TAC AAG ACC  
40 Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr

157 ACA CCT CCC ATG CTG GAC TCC GAC GGC TCC TTC TTC CTC  
53 Thr Pro Pro Met Leu Asp Ser Asp Gly Ser Phe Phe Leu

196 TAC AGC AAG CTC ACC GTG GAC AAG AGC AGG TGG CAG CAG  
66 Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln

235 GGG AAC GTC TTC TCA TGC TCC GTG ATG CAT GAG GCT CTG  
79 Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu

274 CAC AAC CAC TAC ACG CAG AAG AGC CTC TCC CTG TCT CCG  
92 His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro

313 AGT AAA GGT ACC CAG CTC TTC CAC CTA CAG AAG GAG CTG  
105 Ser Lys Gly Thr Gln Leu Phe His Leu Gln Lys Glu Leu

352 GCA GAA CTC CGA GAG TCT ACC AGC CAG ATG CAC ACA GCA  
118 Ala Glu Leu Arg Glu Ser Thr Ser Gln Met His Thr Ala

391 TCA TCT TTG GAG AAG CAA ATA GGA TCC CCC AGT CCA CCC  
131 Ser Ser Leu Glu Lys Gln Ile Gly Ser Pro Ser Pro Pro

430 CCT GAA AAA AAG GAG CTG AGG AAA GTG GCC CAT TTA ACA  
144 Pro Glu Lys Lys Glu Leu Arg Lys Val Ala His Leu Thr

469 GGC AAG TCC AAC TCA AGG TCC ATG CCT CTG GAA TGG GAA  
157 Gly Lys Ser Asn Ser Arg Ser Met Pro Leu Glu Trp Glu

508 GAC ACC TAT GGA ATT GTC CTG CTT TCT GGA GTG AAG TAT  
170 Asp Thr Tyr Gly Ile Val Leu Leu Ser Gly Val Lys Tyr

**FIG. 6A.****SUBSTITUTE SHEET (RULE 26)**

7/7

547 AAG AAG GGT GGC CTT GTG ATC AAT GAA ACT GGG CTG TAC  
183 Lys Lys Gly Gly Leu Val Ile Asn Glu Thr Gly Leu Tyr

586 TTT GTA TAT TCC AAA GTA TAC TTC CGG GGT CAA TCT TGC  
196 Phe Val Tyr Ser Lys Val Tyr Phe Arg Gly Gln Ser Cys

625 AAC AAC CTG CCC CTG AGC CAC AAG GTC TAC ATG AGG AAC  
209 Asn Asn Leu Pro Leu Ser His Lys Val Tyr Met Arg Asn

664 TCT AAG TAT CCC CAG GAT CTG GTG ATG ATG GAG GGG AAG  
222 Ser Lys Tyr Pro Gln Asp Leu Val Met Met Glu Gly Lys

703 ATG ATG AGC TAC TGC ACT ACT GGG CAG ATG TGG GCC CGC  
235 Met Met Ser Tyr Cys Thr Thr Gly Gln Met Trp Ala Arg

742 AGC AGC TAC CTG GGG GCA GTG TTC AAT CTT ACC AGT GCT  
248 Ser Ser Tyr Leu Gly Ala Val Phe Asn Leu Thr Ser Ala

781 GAT CAT TTA TAT GTC AAC GTA TCT GAG CTC TCT CTG GTC  
261 Asp His Leu Tyr Val Asn Val Ser Glu Leu Ser Leu Val

820 AAT TTT GAG GAA TCT CAG ACG TTT TTC GGC TTA TAT AAG  
274 Asn Phe Glu Glu Ser Gln Thr Phe Phe Gly Leu Tyr Lys

859 CTC TAA  
287 Leu \*\*\*

**FIG. 6B.****SUBSTITUTE SHEET (RULE 26)**

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/03571

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 39/395; C07K 16/46

US CL : 424/134.1; 530/387.3, 350, 402

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/134.1; 530/387.3, 350, 402

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, MEDLINE, EMBASE, BIOSIS  
Search terms: fas, ligand

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	TANAKA, M. Expression of the Functional Soluble Form of Human Fas Ligand in Activated Lymphocytes. The EMBO Journal. 1995, Vol. 14, No. 6, pages 1129-1135, see entire document.	1-25
Y	US 4,867,973 A (GOERS et al) 19 September 1989, col. 3 and 14.	1-25
Y	US 5,225,538 A (CAPON et al) 06 July 1993, col. 4.	1-25

Further documents are listed in the continuation of Box C.

See patent family annex.

• Special categories of cited documents:	
•A• document defining the general state of the art which is not considered to be of particular relevance	•T• later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
•E• earlier document published on or after the international filing date	•X• document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
•L• document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reasons (as specified)	•Y• document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
•O• document referring to an oral disclosure, use, exhibition or other means	•&• document member of the same patent family
•P• document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

05 JUNE 1997

Date of mailing of the international search report

20.08.1997

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Faxsimile No. (703) 305-3230

Authorized officer

EMMA CECH

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)\*